Mitochondrial DNA analyses reveal widespread tardigrade diversity in Antarctica

Alejandro Velasco-Castrillón , Sandra J. McInnes , Mark B. Schultz , María Arróniz-Crespo , Cyrille A. D'Haese , John A. E. Gibson , Byron J. Adams Timothy J. Page , Andrew D. Austin , Steven J. B. Cooper and Mark I. Stevens

Abstract. Antarctica contains some of the most challenging environmental conditions on the planet due to freezing temperatures, prolonged winters and lack of liquid water. Whereas 99.7% of Antarctica is permanently covered by ice and snow, some coastal areas and mountain ridges have remained ice-free and are able to sustain populations of microinvertebrates. Tardigrades are one of the more dominant groups of microfauna in soil and limno-terrestrial habitats, but little is known of their diversity and distribution across Antarctica. Here, we examine tardigrades sampled from across an extensive region of continental Antarctica, and analyse and compare their partial mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene sequences with those from the Antarctic Peninsula, maritime and sub-Antarctica, Tierra del Fuego and other worldwide locations in order to recognise operational taxonomic units (OTUs). From 439 new tardigrade *COI* sequences, we identified 98 unique haplotypes (85 from Antarctica) belonging to *Acutuncus*, *Diphascon*, *Echiniscus*, *Macrobiotus*, *Milnesium* and unidentified Parachela. Operational taxonomic units were delimited by Poisson tree processes and general mixed Yule coalescent methods, resulting in 58 and 55 putative species, respectively. Most tardigrades appear to be locally endemic (i.e. restricted to a single geographic region), but some (e.g. *Acutuncus antarcticus* (Richters, 1904)) are widespread across continental Antarctica. Our molecular results reveal: (i) greater diversity than has previously been appreciated with distinct OTUs that potentially represent undescribed species, and (ii) a lack of connectivity between most OTUs from continental Antarctica and those from other Antarctic geographical zones.

Additional keywords: biodiversity, biogeography, *COI* gene, cosmopolitan, endemic, OTUs, refugia, species delimitation, Tardigrada.

Introduction

Tardigrades are a major component of Antarctic soils and one of the most dominant groups of metazoans found in harsh polar environments (Sohlenius *et al.* 1996; Sohlenius and Boström 2008; Convey *et al.* 2008). Despite their abundance in Antarctica, little is known about their diversity and distribution. It has been suggested that Antarctic terrestrial fauna could have survived glaciation in ice-free areas and some might be remnants of fauna

from the Gondwanan super-continent (Stevens *et al.* 2006; Convey and Stevens 2007; Convey *et al.* 2008). However, studies that explore extinction and recolonisation, or persistence in refugia, rarely include comprehensive geographic sampling of Antarctic taxa (e.g. Stevens and Hogg 2006). The harsh conditions of continental Antarctica have restricted microfaunal research largely due to the local environs of research stations and, thus, morphology-based tardigrade studies has been limited in geographic scope and probably provide inaccurate data on their continent-wide distribution (e.g. Sohlenius and Boström 2005; Adams *et al.* 2006).

There are numerous cases of misclassification and underestimation of diversity for most microfaunal groups from Antarctica, likely due to poor taxonomic resolution caused by insufficient sampling (Adams *et al.* 2006). Comprehensive studies from continental Antarctica are lacking and, currently, only a few studies have discussed tardigrades (e.g. Murray 1910; Dastych 1984; Miller *et al.* 1994; Miller and Heatwole 1995; McInnes and Pugh 1998; Convey and McInnes 2005; Pilato and Binda 2010). An integrated taxonomic approach (molecular and morphological; e.g. Stevens *et al.* 2011) is more likely to assist in the recognition of cryptic species of tardigrade and provide valuable data for assessing the distributions of species (e.g. Sands *et al.* 2008*a*; Czechowski *et al.* 2012).

Records of tardigrade species based on morphological taxonomy have been subject to frequent change as relevant taxonomic characters are explored (e.g. Bertolani and Rebecchi 1993; Marley *et al.* 2011). Studies incorporating molecular data have allowed an examination of species hypotheses based on phenotypic or geographical similarities and have revealed complexes of cryptic species (e.g. Bertolani *et al.* 2014). In tardigrades, one of the most notorious problem species, *Macrobiotus hufelandi* Schultze, 1834, which has a global distribution, is now thought to represent a species complex, based on variation in morphology and the cytochrome *c* oxidase subunit I (*COI*) gene (Bertolani *et al.* 2011, 2014). *Milnesium tardigradum* Doyère, 1840 *sensu lato* has also been considered for many years to be a cosmopolitan species with a pan-Antarctic distribution (e.g. Dastych 1984; Miller *et al.* 1994; Miller and Heatwole 1995). Recently, *Milnesium tardigradum sensu stricto* has been redescribed (Michalczyk *et al.* 2012), and with several new species, including *Milnesium antarcticum* Tumanov, 2006 (Tumanov 2006; Smykla *et al.* 2012) from King George Island, indicates that there are likely to be numerous other species on the Antarctic continent.

Sampling close to research stations creates a clear bias for understanding species' distribution patterns and the biogeography of Antarctica. For example, the maritime Antarctic Graham sector and sub-Antarctic islands (Fig. 1) contain the greatest reported tardigrade diversity, but are also the most studied areas (Convey and McInnes 2005). Currently, 65 tardigrade species have been reported from Antarctica, 41 from continental Antarctica, and 20 shared between continental and maritime Antarctica (Table S1 in Supplementary material). Species' overlap between continental Antarctica and the Antarctic Peninsula seems to be more apparent for tardigrades than for other Antarctic microfauna (Pugh 1993; Andrássy 1998), although this has only been recently examined using molecular markers (Czechowski *et al.* 2012). An important aspect to understanding a species' distribution within Antarctica is to be able to recognise the same species occurring across geographic barriers and outside Antarctica. Compounding this issue are two competing assumptions: first, that dispersal capabilities of tardigrades are low (McInnes and Pugh 1998) and therefore species could be recognised as different if they are geographically distant; and second, that long-range dispersal via air currents, or transportation by larger animals might be possible (Marshall and Pugh 1996). However, the result of misidentifications can prevent species in Antarctica (and elsewhere) from being accurately identified as endemic versus cosmopolitan (McInnes 1995) and therefore affect our ability to accurately test these assumptions.

Molecular studies using rRNA genes or spacers (e.g. *18S*, ITS) and other nuclear genes (Sands *et al.* 2008*a*; Robeson *et al.* 2009; Wełnicz *et al.* 2011; Czechowski *et al.* 2012) have been used for species delimitation of tardigrades, but to date these ribosomal markers have either lacked resolution to separate closely related taxa or have been difficult to optimise (e.g. Sands *et al.* 2008*b*; Guidetti *et al.* 2009). Here, we targeted a 658-bp fragment of the mitochondrial *COI* gene that has demonstrated good resolution for separating closely related species in numerous groups (e.g. Hebert *et al.* 2003*a*; Costa *et al.* 2007). This gene is also one of the most regularly used markers in phylogeography and has been implemented as the 'barcoding gene' for metazoans (Hebert *et al.* 2003*b*; Stevens and Hogg 2003; Ashton *et al.* 2008). More recently, *COI* has been used for integrated taxonomy of tardigrades and highlighting potential cryptic speciation (e.g. Cesari *et al.* 2009; Guidetti *et al.* 2009; Czechowski *et al.* 2012).

Delimiting species using statistically based methods, such as the general mixed Yule coalescent (GMYC) (Pons *et al.* 2006) and Poisson tree processes (PTP) (Zhang *et al.* 2013), has been used for invertebrates (e.g. Blanco-Bercial *et al.* 2014; Modica *et al.* 2014; Soldati *et al.* 2014; Velasco-Castrillón *et al.* 2014*a*). The GMYC method infers species boundaries using the branching rates in a dichotomous and rooted ultrametric tree (Modica *et al.* 2014), whereas the PTP method does not require ultrametric trees and models branching events in terms of number of substitutions, requiring only the input of a phylogram (Soldati *et al.* 2014).

In this study, we used the *COI* gene to: (1) investigate the diversity and distribution of tardigrades from across Antarctica; (2) establish operational taxonomic units (OTUs) based on different species delimitation methods; and (3) assess whether identified OTUs occur solely in continental Antarctica or are shared with those from maritime Antarctica, Tierra del Fuego or other worldwide locations.

Materials and methods

Sampled areas

In order to ascribe geographic diversity, and for practical reasons, we divided Antarctica into the following geographical zones: continental Antarctica, maritime Antarctica and sub-Antarctica. Continental Antarctica included the sectors Maud, Enderby, Wilkes, Scott, Byrd and Ronne (as per Pugh 1993) (Fig. 1); maritime Antarctica included the Antarctic Peninsula (Palmer and Graham sectors), South Orkney Islands and South Shetland

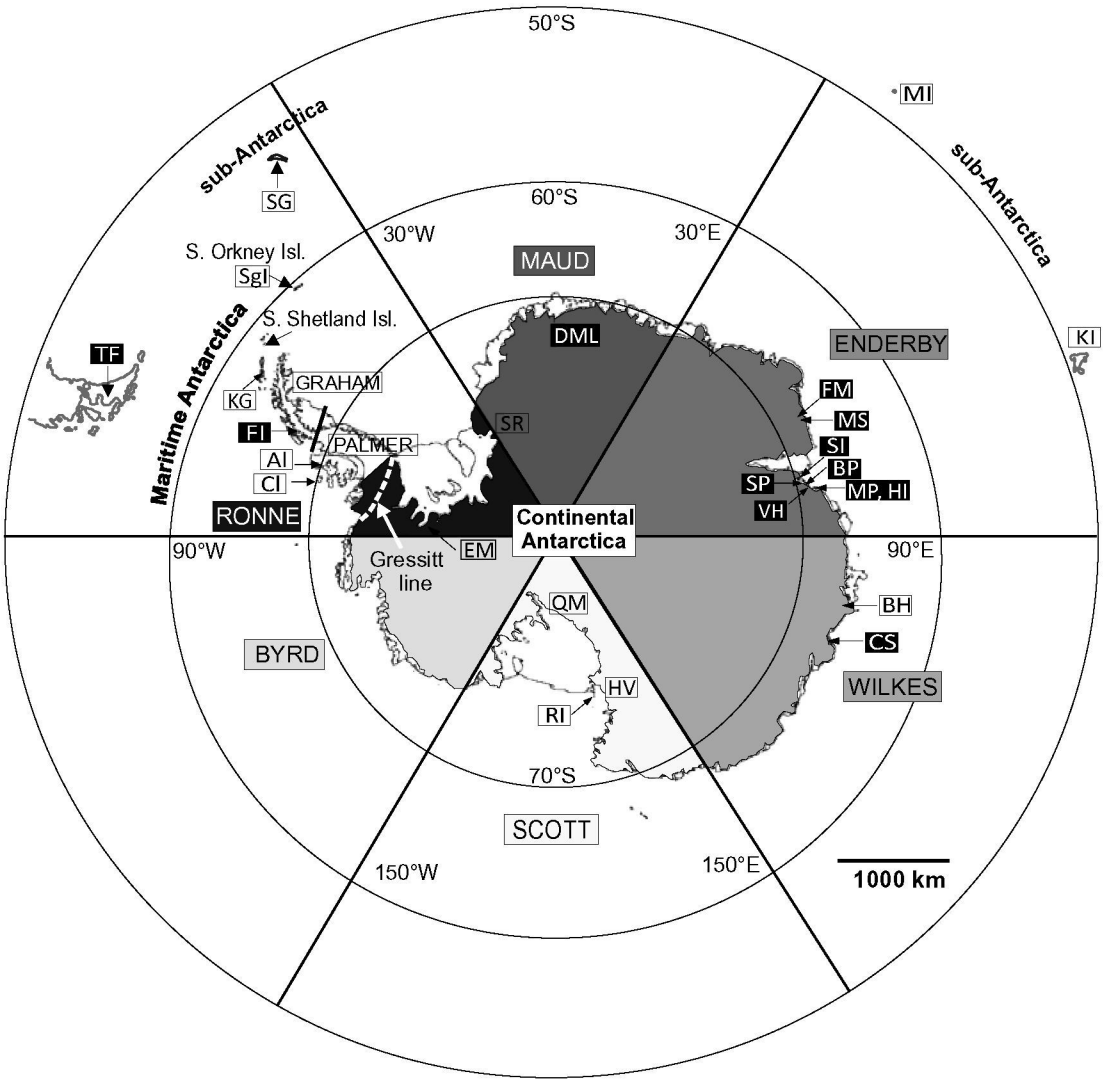

Fig. 1. Map showing six sectors for continental Antarctica (shaded in grey), the Antarctic Peninsula (Graham and Palmer sectors), maritime Antarctica and sub-Antarctic islands. Locations where sampling occurred shown inside black boxes: CS, Casey Station; VH, Vestfold Hills; HI, Hop Island; MP, Mather Peninsula; BP, Broknes Peninsula; SP, Stornes Peninsula; SI, Sansom Island; MS, Mawson Station; FM, Framnes; DML, Dronning Maud Land; FI, Francis Island; TF, Tierra del Fuego in South America. We have also included southern hemisphere regions (for sequences unpublished and from GenBank) that were used in our study: KI, Kerguelen Island; MI, Marion Island; SG, South Georgia; SgI, Signy Island; KG, King George Island; AI, Alexander Island; CI, Charcot Island; EM, Ellsworth Mtns; SR, Shackleton Range; QM, Queen Maud Mtns; HV, Hidden Valley; RI, Ross Island; BH, Bunger Hills.

Islands; and sub-Antarctica included the islands of South Georgia, Kerguelen and Marion (Fig. 1).

Sampling across Antarctica occurred during the summers of 2007 to 2010, covering the Maud, Enderby and Wilkes sectors, and Francis Island off the east coast of Graham Land in the Antarctic Peninsula (Fig. 1). Soil sampling in Wilkes and Enderby sectors was conducted from December 2009 to March 2010, covering a distance of ~2000 km from the Australian base Casey Station on Bailey Peninsula (66.28°S, 110.54°E) to Framnes Mountains (67.77°S, 62.82°E). Tardigrades were extracted from 62 soil samples, collected from Casey Station, Vestfold Hills, Hop Island, Mather Peninsula, Sansom Island, the Australian base Mawson Station, Framnes Mountains, and the Broknes and Stornes peninsulas, both in the Larsemann Hills (Table 1). Aquatic samples were also collected in the Enderby sector (Stornes Peninsula to Vestfold Hills; 76°E–78°E) from 38 permanent waterbodies which ranged in size from small tarns (3 m in diameter) to large lakes (>450 m in diameter). Four soil samples were collected from Francis Island during the summer of 2007–08, and eight soil samples from Tanngarden and Brattnipane in Dronning Maud Land in February 2009 (Table 1). To expand the geographic sampling area, we included tardigrades from 11 soil samples collected from recently deglaciated areas of Tierra del Fuego (54.4°S, 69°W).

Table 1. Sampled areas and locations

Regions sampled from Antarctica (Wilkes, Enderby, Maud and Palmer sectors) and South America (Tierra del Fuego) showing the number of tardigrade samples collected and number of samples successfully sequenced for cytochrome *c* oxidase subunit 1; PCRs, polymerase chain reactions; s, soil sample; w, water sample

Sector	Region	Coordinates		Sampling area or transect	Elevation(m)	Samples extracted	Positive tardigrade PCRs
		Latitude	Longitude				
Wilkes	Casey Station	66.28°S	110.52°–110.54°E	1.5 km^2	4–44	11s	11s
Enderby	Vestfold Hills	68.48°–68.60°S	77.87°–78.51°E	340 km^2	4–66	10s, 4w	5s, 3w
Enderby	Broknes Peninsula	69.38°–69.4°S	76.32°–76.40°E	7 km^2	0–69	6s, 16w	5s, 10w
Enderby	Stornes Peninsula	69.36°–69.43°S	75.99°–76.14°E	6 km^2	4–59	15s, 15w	10s, 14w
Enderby	Hop Island	68.82°–68.83°S	77.68°–77.73°E	4 km^2	10–36	6s, 2w	5s
Enderby	Mather Peninsula	68.85°–68.86°S	77.93°–77.94°E	1 km^2	44–80	3s	1s
Enderby	Sansom Island	69.71°S	73.75° E	400 m^2	15–20	3s	3s
Enderby	Mawson station	67.60°S	62.86°–62.87°E	0.48 km^2	4–24	6s	5s
Enderby	Framnes Mountains	67.77°–67.78°S	62.79°–62.82°E	3 km^2	460–490	2s	0
Maud	Dronning Maud Land	71.97°–72.10°S	23.83°–23.47°E	250 km^2	1253–1387	8s	7s
Palmer	Francis Island	69.59°–69.66°S	64.37°–65.40°W	150 km^2	114–427	4s	1s
South America	Tierra del Fuego	54.68°–54.77°S	69.39°–69.59°W	17 km	10–220	11s	6s

Sampling methods

The top 0–10 cm of soil was excavated as previous studies have shown that the majority of invertebrates inhabit this layer (Powers *et al.* 1995). Each soil sample (0.5–1.0 kg) was excavated using a metal trowel (wiped clean after each use to avoid cross-contamination between sites) and transferred to sterile Whirl-pak bags (1.24 L) which were subsequently stored at −20°C to −80°C. Sample sites were across diverse elevations (from 0 m to 1389 m above sea level, asl; Table 1), a range of vegetation types, and soils of different particle size and geochemical properties (after Velasco-Castrillón *et al.* 2014*b*) to maximise capture of a wide sampling of biodiversity from the terrestrial environment.

Aquatic samples from the Enderby sector were obtained using a throw-net with a 30 cm diameter frame and 35-μm mesh that drained into a removable 50-mL tube. The net, tethered by a 10-m handline, was cast into the waterbody and retrieved quickly to keep it from sinking. The sample from the feed side of the net was washed into and stored in the attached 50-mL tube and suspended in water from the sampled lake.

Sorting and identification

Tardigrades from continental Antarctic soil samples were extracted using an adapted version of a sugar centrifugation protocol (Freckman and Virginia 1993). Extractions were carried out on 50–100-g soil samples (wet weight) following methods described in Velasco-Castrillón *et al.* (2014*b*). Tardigrades from aquatic (initially stored in 50-mL tubes) and soil samples captured on 38-μm mesh were placed in a Petri dish and examined under a stereo dissecting microscope (Olympus SZ-PT, Japan). Selected tardigrade specimens were placed in glass blocks using gel electrophoresis tips. Representatives of each apparent morphotype were transferred with an Irwin loop into a water droplet on a slide positioned under a digital microscope (Celestron, LCD Digital Microscope, USA). Digital images of morphologically selected specimens were taken at 40× to 100× magnification. Each specimen was then transferred to a unique well in 96-well microplates. Unused tardigrades were put back into tubes with 100% ethanol and stored at −20°C.

Morphological identification of specimens was carried out to generic level for *Echiniscus* spp. (order Echiniscoides), *Milnesium* spp. (order Apochela), *Diphascon* spp. and *Macrobiotus* spp., and species level for *Acutuncus antarcticus* (Richters, 1904); the latter three belonging to the order Parachela. Superficial identification of some of the Parachela specimens was only possible from digital images and was therefore left as 'Parachela' (Fig. 2).

Sequence analyses

The *COI* gene was bidirectionally sequenced by the Canadian Centre for DNA Barcoding (CCDB; Appendix S1). Chromatograms were manually inspected and edited in Geneious v. 3.8 (Biomatters, Auckland, NZ) to resolve unclear base calls. Sequences (ranging from 418 to 660 bp) were translated into amino acids using the invertebrate mitochondrial genetic code to check for stop-codons. After confirming the absence of stop-codons in all sequences, they were matched against phylum and published haplotypes using the Barcode of Life Data Systems (BOLD) search engine and the Blastn algorithm implemented in GenBank to confirm sequences were amplified from the target tardigrade DNA. Our sequence data were complemented with previously unpublished tardigrade sequences from a variety of collections made throughout Antarctica and maritime Antarctic islands. Of these, sequenced tardigrade material covers Marion Island, King George Island, South Georgia, Signy Island, Ellsworth Mountains, Queen Maud Mountains and Hidden Valley (Fig. 2). Additionally, tardigrades from the Antarctic Peninsula region, sub-Antarctic islands and Ellsworth Mountains (S. J. McInnes and C. J. Sands, unpubl. data) were morphologically identified to species or 'genus awaiting species description' (*D. puniceum* Jennings, 1971 [An8], *Diphascon* spp. [An1, An3, An4], *Milnesium* spp. [An16, An19], *E. jenningsi* Dastych, 1984 [An23], and *Echiniscus* spp. [An20, An22, An24–An27]) (for accession numbers see Table 2).

Alignments were performed using the default settings of the Geneious alignment algorithm (cost matrix: 65% similarity; gap open penalty: 12; and gap extension penalty: 3). The general

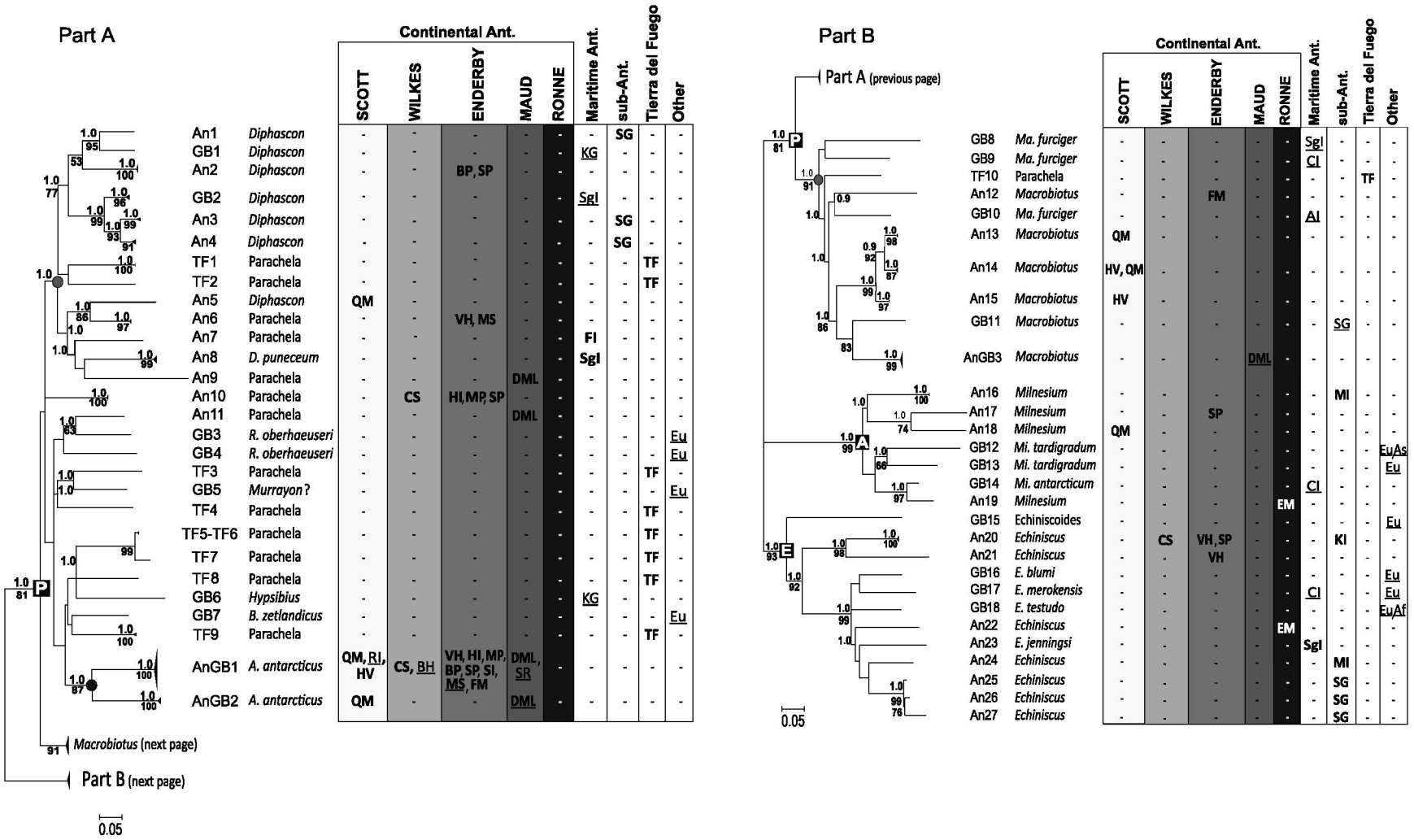

Fig. 2. Maximum likelihood and Bayesian inference dendrogram using cytochrome *c* oxidase subunit 1 tardigrade sequences from continental, maritime and sub-Antarctica, Tierra del Fuego (TF) and other sites (Eu, Europe; As, Asia; Af, Africa). Part A: includes the order Parachela (P). Part B: includes the *Macrobiotus* clade of the order Parachela, and the orders Apochela (A) and Echiniscoidea (E). Orders are indicated by white letters inside black boxes. Bootstrap values from 1000 pseudoreplicates are shown below the node (bootstrap values below 50% are not shown). Posterior probabilities $\geq$0.9 from the Bayesian analysis are shown above the branch. The geographic regions in bold include new sequences from our study. The regions underlined include sequences gathered from GenBank. The solid grey circles indicate the clades for *Diphascon* (and five unidentified Parachela), *A. antarcticus*, *Macrobiotus*, *Milnesium* and Echiniscoides. Scale bar represents substitutions per nucleotide site. Refer to Fig. 1 for region abbreviations.

Table 2. Tardigrade accession numbers (GenBank) for cytochrome *c* oxidase subunit 1 sequences

Accession numbers in bold correspond to haplotypes generated for this study (numbers starting with KJ) or provided by Sands, C. J. and McInnes, S. J. (numbers starting with KP). Accession numbers underlined refer to sequences gathered from GenBank; PTP, Poisson tree processes; OTUs, operational taxonomic units

PTP (OTUs)	Taxa	Accession numbers
An1	*Diphascon*	**KP013606**
GB1	*Diphascon*	EF632530
An2	*Diphascon*	**KJ856916–KJ856920**
GB2	*Diphascon*	EF632531–EF632536
An3	*Diphascon*	**KP013605, KP013607**
An4	*Diphascon*	**KP013608–KP013610**
TF1	Parachela	**KJ856926, KJ856927**
TF2	Parachela	**KJ856928**
An5	*Diphascon*	**KJ856929**
An6	Parachela	**KJ856930–KJ856932**
An7	Parachela	**KJ856933**
An8	*Diphascon puneceum*	**KP013597, KP013598, KP013600, KP013612, KP013614**
An9	Parachela	**KJ856939**
An10	Parachela	**KJ856940–KJ856942**
An11	Parachela	**KJ856943**
GB3	*Ramazzottius oberhaeuseri*	EU251379, EU251380
GB4	*Ramazzottius oberhaeuseri*	FJ435799
TF3	Parachela	**KJ856944**
GB5	*Murrayon?*	FJ435801
TF4	Parachela	**KJ856945**
TF5-TF6	Parachela	**KJ856946–KJ856948**
TF7	Parachela	**KJ856949**
TF8	Parachela	**KJ856950**
GB6	*Hypsibius*	EF632518, EF632519
GB7	*Borealibius zetlandicus*	FJ184601, FJ184602
TF9	Parachela	**KJ856951, KJ856952**
AnGB1	*Acutuncus antarcticus*	**KJ856953–KJ856976,** JX486021–JX486025, JX865305
AnGB2	*Acutuncus antarcticus*	**KJ856977–KJ856979,** JX296185, JX296188–JX296190, JX296198, JX296205, JX296213, JX296215
GB8	*Macrobiotus furciger*	JX865308
GB9	*Macrobiotus furciger*	JX865306
TF10	Parachela	**KJ856980**
An12	*Macrobiotus*	**KJ856981**
GB10	*Macrobiotus furciger*	JX865314
An13	*Macrobiotus*	**KJ856982, KJ856983**
An14	*Macrobiotus*	**KJ856984–KJ856989**
An15	*Macrobiotus*	**KJ856990, KJ856991**
GB11	*Macrobiotus*	JX865310
AnGB3	*Macrobiotus*	**KJ856992–KJ856998,** JX296219, JX296220, JX296222–JX296229, JX296231–JX296233, JX296235, JX296237, JX296238, JX296240–JX296243, JX296246, JX296247, JX296249–JX296251, JX296253–JX296256, JX296260, JX296261, JX296278, JX296282, JX296295, JX296306, JX296312, JX296313, JX296358, JX296359, JX296361, JX888911
An16	*Milnesium*	**KP013598, KP013601**
An17	*Milnesium*	**KJ857001**
An18	*Milnesium*	**KJ857002**
GB12	*Milnesium tardigradum*	EU244603, EU244604
GB13	*Milnesium tardigradum*	JN664950
GB14	*Milnesium tardigradum*	EF632553
An19	*Milnesium*	**KP013613**
GB15	Echiniscoides	JF437735–JF437742, JF437744, JF437747, JF437749–JF437752, JF437755, JF437757, JF437764–JF437766, JF437771, JF437772, JF437775, JF437777, JF437779, JF437782, JF437784
An20	*Echiniscus*	**KJ857004–KJ857007, KP057515**
An21	*Echiniscus*	**KJ857008**

(*continued next page*)

Table 2. (*continued*)

PTP (OTUs)	Taxa	Accession numbers
GB16	*Echiniscus*	EU046090, EU046091, EU046093–EU046100, EU046102–EU046107, EU046109–EU046128, EU046130, EU046131, EU046133–EU046140, EU046145–EU046160, EU046162–EU046165, EU046172, EU046175, EU046176, EU046178–EU046180, EU046183–EU046196, FJ435815
GB17	*Echiniscus merokensis* + *Echiniscus* (unnamed)	EF632539, EF632541
GB18	*Echiniscus testudo*	EF620377, EF620378, EF632542, EF632543, EF630370–EF630372, EF630374, EU244601
An22	*Echiniscus corrugicaudatus*	**KP057514**
An23	*Echiniscus jenningsi*	**KP013596**
An24	*Echiniscus*	**KP013604**
An25	*Echiniscus*	**KP013602**
An26	*Echiniscus*	**KP013603**
An27	*Echiniscus*	**KP013611**

time reversible model with invariant sites and gamma distribution (GTR+I+Γ) was chosen as the best model of sequence evolution by Modelltest v. 3.7 (Posada and Crandall 1998), under the Akaike information criterion (AIC) run in PAUP* v. 4.0b10 (Swofford 2002). Maximum likelihood (ML) using a GTR+I+Γ model and 1000 bootstrap pseudoreplicates was implemented using MEGA5 (Tamura *et al.* 2011). Bayesian inference used a GTR+I+Γ model in MrBayes v. 3.2 (Huelsenbeck and Ronquist 2001) with two replicates, four chains per replicate (three heated and one cold chain), 5 000 000 generations per run (to result in an average standard deviation of split frequencies below 0.01), a chain temperature of 0.2, sampling frequency of 100, and a burn-in of 25% (12 500 discarded trees). Sequences from species from the order Echiniscoidea were used as outgroups in the Bayesian analysis.

We calculated pair-wise distances among haplotypes using uncorrected p-distances (implemented in MEGA5; Tamura *et al.* 2011; Table S2), as studies have shown p-distances to be a useful way to delimit species and/or OTUs (Srivathsan and Meier 2012). Operational taxonomic units were inferred by PTP and GMYC models (Table 3) implemented in Python using the Tree Exploration Package (Huerta-Cepas *et al.* 2010). For the PTP model, the original ML tree generated in MEGA5 was used as input. For GMYC, an ultrametric tree was generated in BEAST 1.6.2 (Drummond and Rambaut 2007) using the following parameters: a GTR+I+Γ evolutionary model, a relaxed clock (uncorrelated lognormal), and the Yule process speciation prior. The analysis was run for 100 million generations with parameters sampled every 2000 generations and a burn-in value of 20%. A tree file was generated and visualised in FigTree 1.3.1 (Rambaut and Drummond 2009*b*) and then OTUs were delimited using the GMYC model. The log file (produced in BEAST) was analysed in Tracer 1.5.0 (Rambaut and Drummond 2009*a*) to assess for convergence (by visual inspection of histograms for normal distribution).

The *COI* sequences obtained in this study have been deposited in GenBank (Table 2) and in the BOLD systems (www.barcodinglife.org), together with metadata for each voucher specimen. Detailed specimen records, collection data and sequence information, including trace files and primer details are accessible in BOLD in the ANTAR (Antarctic Invertebrates) project.

Results

Molecular diversity

We generated 439 sequences of length 418–658 bp which corresponded to 98 haplotypes (Table 3). Of these, 379 sequences were from continental Antarctica, 42 were from maritime and sub-Antarctica and 18 were from Tierra del Fuego. We included publicly available GenBank sequences from Antarctica and worldwide locations (32 unique haplotypes) which all had pair-wise p-distance divergence of less than 29% to any of our 98 haplotypes. In total, 58 and 55 OTUs were determined by the PTP and GMYC models, respectively. The GMYC model grouped three of the PTP-OTUs from Tierra del Fuego (TF5, TF6 and TF7) and two from sub-Antarctica (An25 and An26) as two separated OTUs (Table 3). We found intra-OTU p-distance divergence (intra-div) ranging between 0 and 4.6%. The minimum inter-OTU p-distance divergence (inter-div) was observed for TF5 and TF6 (0.5% divergence). Of those OTUs estimated by PTP, 37 were formed exclusively by unique haplotypes generated from this study (Antarctic OTUs An1–An27 and Tierra del Fuego OTUs TF1–TF10). Three OTUs (AnGB1–AnGB3) comprised haplotypes obtained in this study and haplotypes from GenBank, and 20 OTUs exclusively comprised haplotypes from GenBank (GB1–GB18) (Table 3).

Order Parachela

Parachela was represented by 39 PTP-OTUs, of which 15 could not be identified to genus or species rank (indicated as 'Parachela'; Fig. 2; Table 3). Operational taxonomic units were mostly locally endemic (i.e. restricted to a single sector; Fig. 2) except for *A. antarcticus* (AnGB1 and AnGB2) and one unidentified Parachela OTU (An10; Table 3). An10 included haplotypes from specimens collected from Wilkes and Enderby sectors (covering a distance of ~1500 km). *Acutuncus antarcticus* was the most common tardigrade throughout continental Antarctica; it formed two highly divergent OTUs, AnGB1 and AnGB2 (up to 20.4% inter-div; Table S2) that likely represent two separate species. AnGB1 (up to 1.6% intra-div) occurred in terrestrial and aquatic samples and across the widest geographical area, including the sectors Maud, Enderby, Wilkes and Scott. AnGB2 (up to 1.4%

Table 3. Operational taxonomic units (OTUs) estimated by the delimitation models Poisson tree processes (PTP) and general mixed Yule coalescent (GMYC) models

Operational taxonomic units An1–An27 (Antarctica) and TF1–TF10 (Tierra del Fuego) were formed exclusively by haplotypes from this study; OTUs AnGB1–AnGB3 combine haplotypes generated from this study and GenBank sequences; OTUs GB1–GB18 include only haplotypes recovered from GenBank (sequences and haplotypes obtained from GenBank are shown in square brackets); solid boxes indicate two or more PTP-OTUs forming one GMYC-OTU; w, sequences from specimens collected from water samples; intra-div, intra-OTU p-distance divergence

Taxa	No. of sequences	No. of haplotypes	Intra-div (%)	PTP (OTUs)	GMYC (OTUs)
Diphascon	1	1	0	An1	An1
Diphascon	[1]	[1]	0	GB1	GB1
Diphascon	3, 16w	1, 4w	0–0.5	An2	An2
Diphascon	[6]	[1]	0–2.7	GB2	GB2
Diphascon	2	2	1.8	An3	An3
Diphascon	7	3	0.2–1.4	An4	An4
Parachela	3	2	0.2	TF1	TF1
Parachela	1	1	0	TF2	TF2
Diphascon	1	1	0	An5	An5
Parachela	6	3	0.2–0.7	An6	An6
Parachela	1	1	0	An7	An7
Diphascon puniceum	6	5	0.2–1.4	An8	An8
Parachela	1	1	0	An9	An9
Parachela	34	3	0.003–0.01	An10	An10
Parachela	1	1	0	An11	An11
Ramazzottius oberhaeuseri	[2]	–	<1.0	GB3	GB3
Ramazzottius oberhaeuseri	[1]	[1]	0	GB4	GB4
Parachela	1	1	0	TF3	TF3
Murrayon?	[1]	[1]	0	GB5	GB5
Parachela	3	1	0	TF4	TF4
Parachela	3	3	0–0.5	TF5–TF6	TF5–TF6 + TF7 (inter-div: 3.3–3.4%)
Parachela	1	1	0	TF7	
Parachela	3	1	0	TF8	TF8
Hypsibius	[2]	[1]	0	GB6	GB6
Borealibius zetlandicus	[2]	[1]	0	GB7	GB7
Parachela	2	2	1.4	TF9	TF9
Acutuncus antarcticus	211, 46w [6]	17, 7w [3]	0–1.6	AnGB1	AnGB1
Acutuncus antarcticus	5 [8]	3 [1]	0.2–1.4	AnGB2	AnGB2
Macrobiotus furciger	[1]	[1]	0	GB8	GB8
Macrobiotus furciger	[1]	[1]	0	GB9	GB9
Parachela	1	1	0	TF10	TF10
Macrobiotus	1	1	0	An12	An12
Macrobiotus furciger	[1]	[1]	0	GB10	GB10
Macrobiotus	2	2	0.7	An13	An13
Macrobiotus	10	6	0–3.9%	An14	An14
Macrobiotus	3	2	0.2	An15	An15
Macrobiotus	[1]	[1]	0	GB11	GB11
Macrobiotus	11 [41]	5 [9]	0–4.6%	AnGB3	AnGB3
Milnesium	3	2	0.5	An16	An16
Milnesium	2	1	0	An17	An17
Milnesium	1	1	0	An18	An18
Milnesium tardigradum	[2]	[2]	<1.0	GB12	GB12
Milnesium tardigradum	[1]	[1]	0	GB13	GB13
Milnesium antarcticum	[1]	[1]	0	GB14	GB14
Milnesium	3	1	0	An19	An19
Echiniscoides	[29]	–	<1.0	GB15	GB15
Echiniscus	13	4	0.2–0.5	An20	An20
Echiniscus	2	1	0	An21n	An21n
Echiniscus blumi	[87]	–	<1.0	GB16	GB16
E. merokensis + *Echiniscus* (unnamed)	[2]	–	<1.0	GB17	GB17
Echiniscus testudo	[9]	–	<1.0	GB18	GB18
Echiniscus	9	1	0	An22	An22
Echiniscus jenningsi	1	1	0	An23	An23

(*continued next page*)

Table 3. (*continued*)

Taxa	No. of sequences	No. of haplotypes	Intra-div (%)	PTP (OTUs)	GMYC (OTUs)
Echiniscus	1	1	0	An24	An24
Echiniscus	15	1	0	An25	An25 + An26 (inter-div: 2.5%)
Echiniscus	2	1	0	An26	
Echiniscus	1	1	0	An27	An27
Total	439 [197]	98 [>31]		58	55

intra-div) was more restricted and was present only in Scott and Maud sectors (2600 km apart; Figs 1, 2).

The genus *Diphascon* included OTUs from three different geographical zones. Two OTUs were from continental Antarctica (An2 and An6), three from maritime Antarctica (GB1, GB2 and An8) and three from sub-Antarctica (An1, An3 and An4). When comparing sequence similarity (based on p-distance) between geographical zones, we observed that the closest similarity corresponded to: (i) An1 from sub-Antarctic South Georgia and GB1 from King George Island (12.2% inter-div), 1500 km distant; and (ii) Signy Island GB2 and the South Georgia sub-clade An3–An4 (up to 13% inter-div; Table S2). We also observed that five unidentified Parachela (TF1, TF2, An6, An7 and An9) fell within the *Diphascon* clade and are likely to represent distinct species (Fig. 2; Fig. S1).

Both the Bayesian and the ML trees revealed a well-supported *Macrobiotus* clade (Fig. 2; Figs S1, S2) including nine OTUs (Table 3) of *Macrobiotus* spp. from continental Antarctica (An12–An15), maritime Antarctica (GB8–GB10) and South Georgia (GB11), and one unidentified Parachela OTU from TF (TF10; Fig. 2). The maximum sequence divergence within the *Macrobiotus* clade was between GB8 and An14 (26.8% inter-div; Table S2). The *Macrobiotus* OTUs An13–An15, formed by sequences from Scott sector (within 700 km), consisted of a well-supported sub-clade (5.3–8.2 inter-div; Fig. 2; Figs S1, S2). Surprisingly the single European *Murrayon* GenBank sequence GB6 did not group within the Macrobiotoidea, and is most likely the result of morphological identification or a typographic entry error.

Order Apochela

The monogeneric order Apochela formed a well-supported monophyletic clade (Fig. 2; Figs S1, S2) showing seven *Milnesium* OTUs (8.2–25.4% inter-div; Table S2). Three of these OTUs (GB12–GB14) were exclusively formed by GenBank sequences that represented the nearest matches for the four *Milnesium* OTUs found in this study. GenBank labelled *Mi. tardigradum* GB12 (from Germany and Japan) and GB13 (from Germany; 19.7% inter-div) represented the recently redescribed *Mi. tardigradum sensu stricto* (Michalczyk *et al.* 2012). The third GenBank haplotype, *Mi. antarcticum* GB14, from Palmer sector in the Antarctic Peninsula, was the closest match to a single OTU haplotype (An19, 8.2% inter-div) from Ronne sector, 1000 km away. Three unidentified *Milnesium* OTUs An16–An18 (18.5–23.6% inter-div) were from Marion Island An16 and continental Antarctica An17–An18 (~2400 km distant).

Order Echiniscoidea

Echiniscoidea was represented by an unidentified European GenBank sequence of *Echiniscoides* (GB15) and 11 *Echiniscus* OTUs (An20–An27 and GB16–GB18; 2.5–31% inter-div). GenBank OTUs (16–18% inter-div; Table S2) grouped in three OTUs: European *Echiniscus blumi* Richters, 1903 (GB16), European–African *E. testudo* (Doyère, 1840) (GB18), and European *E. merokensis* Richters, 1904 and an unnamed maritime Antarctic *Echiniscus* (GB17). The latter differs morphologically from *E. merokensis*, so further investigation of its species status is warranted.

Two highly divergent *Echiniscus* OTUs (An20 and An21; 21% inter-div) showed a 3-bp deletion (position 292–294 bp) compared with the tardigrade alignment. We found this 3-bp position to be highly variable in all *Echiniscus* haplotypes, corresponding to the amino acids valine (An22, An24 and GB18), alanine (An23, An25, An26 and An27), isoleucine (GB16) and serine (GB17). Operational taxonomic unit An20 (0.2–0.5% intra-div) comprised samples from continental Antarctica (Wilkes and Enderby sectors) and sub-Antarctic Kerguelen Island (~3000 km distant). The closest *Echiniscus* OTUs (An25–An27) were formed by a South Georgian sub-clade (2.5–6.2% inter-div; Table S2). Less closely aligned with this sub-clade was *E. jenningsi* OTU (An24) from Marion Island (14.6–17.2% inter-div) 6600 km away.

Discussion

Operational taxonomic unit delimitation

Using the *COI* gene to establish species boundaries is problematic because different thresholds have been used for different groups (e.g. Bertolani and Rebecchi 1993; Jørgensen *et al.* 2007; Cesari *et al.* 2009; Czechowski *et al.* 2012; Vicente *et al.* 2013). If we consider our OTUs as putative species we find that the intra-OTU p-distance divergence varies greatly among taxa. The highest p-distance divergence within any of our delimited putative species (determined by both the PTP and GMYC models) was observed for continental Antarctic *Macrobiotus* AnGB3 (0–4.6% intra-div) and An14 (0–3.9% intra-div), followed by Signy Island *Diphascon* GB2 (0–2.7% intra-div). When comparing the number of OTUs delimited by PTP and GMYC, we found that the latter model was more conservative, recognising TF5–TF7 as one Parachela species (up to 3.4% inter-div) and An25–An26 as one *Echiniscus* species (2.5% inter-div). Nonetheless, neither of these two sub-clades exceeded the maximum p-distance divergence shown by *Macrobiotus* AnGB3 (4.6% intra-div).

Species diversity

Many Antarctic microfauna are currently considered endemic (e.g. Andrássy 1998) and recent studies have indicated greater species diversity than previously reported. An example is a recent integrated taxonomic study of Antarctic nematodes, which found a combination of locally restricted species and those with broader Antarctic distributions (Velasco-Castrillón and Stevens 2014).

A recent study on Antarctic tardigrades used molecular OTUs to indicate potential new and endemic species within the genera *Macrobiotus* and *Acutuncus* (Czechowski *et al.* 2012). When combined with our sequences, three OTUs for continental Antarctica were identified, *A. antarcticus* AnGB1 and AnGB2, and *Macrobiotus* OTU AnGB3 (Table 3). Even though sequence data were previously available in GenBank for these three OTUs, no records were present for AnGB1 in Maud sector or for AnGB2 in Scott sector (Fig. 2). When considering other taxa we found that only five species of *Echiniscus* have been confirmed by α taxonomy from continental, maritime and sub-Antarctica (Table S1); while our results show nine *Echiniscus* OTUs are present, eight of which were recognised exclusively by sequences generated in this study (Fig. 2). Although *Mi. antarcticum* has only been reported from the maritime Antarctic and *Mi. tardigradum sensu lato* from various Antarctic and sub-Antarctic sites (Table S1), our study has revealed there are five distinct *Milnesium* OTUs (four of which are new records) spread across the Antarctic and sub-Antarctic (Fig. 2). For the genus *Diphascon* we compiled data for 12 species reported in Antarctica (Table S1); however, we were only able to identify six OTUs recognised exclusively by sequences from this study (Table 3).

We are faced with a dilemma when trying to match tardigrade sequences with known Antarctic species identified using α taxonomy (Table S1). Most previously reported species do not have sequences available in GenBank or BOLD (e.g. *Diphascon dastychi* Pilato & Binda, 1999; *D. sanae* Dastych, Ryan & Watkins, 1990; *D. victoriae* Pilato & Binda, 1999; *Macrobiotus blocki* Dastych, 1984; *M. polaris* (Murray, 1910); *M. krynauwi* Dastych & Harris, 1995; *M. meridionalis* Richters, 1909; *Echiniscus corrugicaudatus* McInnes, 2010; *E. pseudowendti* Dastych, 1984; *E. jenningsi*). Therefore, it is currently unclear whether our OTUs from the genera *Diphascon*, *Macrobiotus* and *Echiniscus* correspond to new, undescribed species, or to previously described species (Table S1).

Geographic distribution

Determining the geographic range of species using molecular data provides insights into the potential colonisation of Antarctica from more northern latitudes and the mode of dispersal across the Antarctic landscape (McInnes and Pugh 1998; Stevens *et al.* 2006; Gibson *et al.* 2007; Fontaneto *et al.* 2008). Tardigrades have light-weight dormant life history stages that have been reported in aerial plankton (Kristensen 1987) and are therefore likely to be transported by air currents, potentially assisting dispersal inland (e.g. McInnes and Pugh 1998; Nkem *et al.* 2006; Sohlenius and Boström 2008; Hengherr *et al.* 2010), as suggested for some Antarctic rotifer and nematode species (Nkem *et al.* 2006; Velasco-Castrillón *et al.* 2014*a*; Velasco-Castrillón and Stevens 2014). Parthenogenetic reproduction, described in some tardigrades (Pilato and Binda 2001), might also provide a greater chance of colonising new environments because only a single specimen is required to start a population. Considering the biology of tardigrades and their potential dispersal capabilities we could expect wider species' distributions across Antarctica and, thus, it is surprising that most potential species (i.e. OTUs) appear to be locally endemic. A possible explanation to understand the lack of overlapping species between continental Antarctica and the Antarctic Peninsula is the geographical discontinuity (the Gressitt Line) that separates these two previously identified biogeographic zones (see Chown and Convey 2007) (Fig. 1).

When looking at closely related sequences between tardigrades south of 60°S (continental and maritime Antarctica) and those north of 60°S (sub-Antarctica; Fig. 1) the highest similarity was seen for: (i) *Echiniscus* OTU An20, which comprised haplotypes in both continental Antarctica (Wilkes and Enderby sectors) and in the sub-Antarctic Kerguelen Island; and (ii) the *Diphascon* sub-clade formed by maritime Antarctic GB2 (from Signy Island) and sub-Antarctic An3–An4 (from South Georgia; 10.3–13% inter-div). When continental and maritime Antarctica were compared we could not find shared OTUs among these geographical zones. However, Antarctica is under-sampled and there are large ice-free regions with limited data (e.g. McGaughran *et al.* 2011). This, coupled with the restricted information available for Antarctic tardigrades in GenBank, means that there is potential for bias when interpreting their biogeography and Antarctic endemism.

The distribution of Antarctic tardigrade OTUs has revealed contrasting biogeographic patterns. Most putative species were found to be locally restricted to a particular geographical zone. Those endemic to continental Antarctica were mostly exclusive to a particular sector and none were shared with the Antarctic Peninsula. We have also shown that there are currently more putative species for the genera *Acutuncus*, *Milnesium* and *Echiniscus* than has been reported (morphologically) for Antarctica. While emphasising that our current knowledge is still extremely limited, our data provide further insights into the scale of tardigrade diversity across Antarctica.

Acknowledgements

We would like to thank three anonymous reviewers and Łukasz Kaczmarek for their valued feedback for improving the manuscript. This research was partly funded and supported by the Australian Antarctic Division (http://www.antarctica.gov.au/) Project (ASAC Project 2355 to MIS). We would like to thank The University of Adelaide (http://www.sciences.adelaide.edu.au/) for a PhD scholarship to AVC and the South Australian Museum Mawson Trust for providing funding for the Sir Douglas Mawson Doctoral Scholarship (http://www.samuseum.sa.gov.au/). We deeply thank the Australian Antarctic Stations' leaders and personnel who contributed to logistics and support of our field work in Antarctica. We also thank Rebecca Stonor, Maria Manjarrez, Pauline Glocke, Tanya Matic (The University of Adelaide) and Federica Colombo for their ongoing laboratory support. We also appreciate the assistance with phylogenetic packages given by Mohammad Javidkar. We are grateful to the DNA sequencing agencies BOLD and the CCDB. We would like to thank Peter Convey, Paul Geissler (British Antarctic Survey) for collecting and providing soil samples from Antarctic Peninsula, and to Allan Green, Leopoldo Sancho and Ana Pintado (Complutense University of Madrid) for supplying soil samples from TF and laboratory facilities. We are

extremely grateful to Chester Sands for reviewing the paper and providing feedback, for facilitating soil samples from AP and Dronning Maud Land and for providing sequences from Ronne sector, maritime and sub-Antarctica. All samples were collected under appropriate collection permits and approved ethics guidelines.

References

Adams, B. J., Bardgett, R. D., Ayres, E., Wall, D. H., Aislabie, J., Bamforth, S., Bargagli, R., Cary, C., Cavacini, P., Connell, L., Convey, P., Fell, J. W., Frati, F., Hogg, I. D., Newsham, K. K., O'Donnell, A., Russell, N., Seppelt, R. D., and Stevens, M. I. (2006). Diversity and distribution of Victoria Land biota. *Soil Biology & Biochemistry* **38**, 3003–3018. doi:10.1016/j.soilbio.2006.04.030

Andrássy, I. (1998). Nematodes in the sixth continent. *Journal of Nematode Morphlogy and Systematics* **1**, 107–186.

Ashton, G. V., Stevens, M. I., Hart, M. C., Green, D. H., Burrows, M. T., Cook, E. J., and Willis, K. J. (2008). Mitochondrial DNA reveals multiple northern hemisphere introductions of *Caprella mutica* (Crustacea, Amphipoda). *Molecular Ecology* **17**, 1293–1303. doi:10.1111/j.1365-294X.2007.03668.x

Bertolani, R., and Rebecchi, L. (1993). A revision of the *Macrobiotus hufelandi* group (Tardigrada, Macrobiotidae), with some observations on the taxonomic characters of eutardigrades. *Zoologica Scripta* **22**, 127–152. doi:10.1111/j.1463-6409.1993.tb00347.x

Bertolani, R., Biserov, V., Rebecchi, L., and Cesari, M. (2011). Taxonomy and biogeography of tardigrades using an integrated approach: new results on species of the *Macrobiotus hufelandi* group. *Invertebrate Zoology* **8**, 23–36.

Bertolani, R., Guidetti, R., Marchioro, T., Altiero, T., Rebecchi, L., and Cesari, M. (2014). Phylogeny of Eutardigrada: new molecular data and their morphological support lead to the identification of new evolutionary lineages. *Molecular Phylogenetics and Evolution* **76**, 110–126. doi:10.1016/j.ympev.2014.03.006

Blanco-Bercial, L., Cornils, A., Copley, N., and Bucklin, A. (2014). DNA barcoding of marine copepods: assessment of analytical approaches to species identification. *PLoS Currents* **1**, 6.

Cesari, M., Bertolani, R., Rebecchi, L., and Guidetti, R. (2009). DNA barcoding in Tardigrada: the first case study on *Macrobiotus macrocalix* Bertolani & Rebecchi 1993 (Eutardigrada, Macrobiotidae). *Molecular Ecology Resources* **9**, 699–706. doi:10.1111/j.1755-0998.2009.02538.x

Chown, S. L., and Convey, P. (2007). Spatial and temporal variability across life's hierarchies in the terrestrial Antarctic. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **362**, 2307–2331. doi:10.1098/rstb.2006.1949

Convey, P., and McInnes, S. J. (2005). Exceptional tardigrade-dominated ecosystems in Ellsworth Land, Antarctica. *Ecology* **86**, 519–527. doi:10.1890/04-0684

Convey, P., and Stevens, M .I. (2007). Antarctic biodiversity. *Science* **317**, 1877–1878. doi:10.1126/science.1147261

Convey, P., Gibson, J. A. E., Hillenbrand, C. D., Hodgson, D. A., Pugh, P. J. A., Smellie, J. L., and Stevens, M. I. (2008). Antarctic terrestrial life – challenging the history of the frozen continent? *Biological Reviews of the Cambridge Philosophical Society* **83**, 103–117. doi:10.1111/j.1469-185X.2008.00034.x

Costa, F. O., deWaard, J. R., Boutillier, J., Ratnasingham, S., Dooh, R. T., Hajibabaei, M., and Hebert, P. D. N. (2007). Biological identifications through DNA barcodes: the case of the Crustacea. *Canadian Journal of Fisheries and Aquatic Sciences* **64**, 272–295. doi:10.1139/f07-008

Czechowski, P., Sands, C. J., Adams, B., D'Haese, C., Gibson, J., McInnes, S. J., and Stevens, M. I. (2012). Antarctic Tardigrada: a first step in understanding molecular operational taxonomic units (MOTUs) and biogeography of cryptic meiofauna. *Invertebrate Systematics* **26**, 526–538. doi:10.1071/IS12034

Dastych, H. (1984). The Tardigrada from Antarctic with descriptions of several new species. *Acta Zoologica Cracoviensia* **27**, 377–436.

Dastych, H., and Harris, J. M. (1995). A new species of the genus *Macrobiotus* from inland nunataks in western Dronning Maud Land, continental Antarctica (Tardigrada). *Entomologische Mitteilungen aus dem Zoologischen Museum Hamburg* **11**, 175–182.

Dastych, H., Ryan, G., and Watkins, P. (1990). Notes on Tardigrada from western Dronning Maud Land (Antarctica) with a description of two new species. *Entomologische Mitteilungen aus dem Zoologischen Museum Hamburg* **10**, 57–66.

Drummond, A. J., and Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**, 214. doi:10.1186/1471-2148-7-214

Fontaneto, D., Barraclough, T. G., Chen, K., Ricci, C., and Herniou, E. A. (2008). Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. *Molecular Ecology* **17**, 3136–3146. doi:10.1111/j.1365-294X.2008.03806.x

Freckman, D. W., and Virginia, R. A. (1993). Extraction of nematodes from Dry Valley Antarctic soils. *Polar Biology* **13**, 483–487. doi:10.1007/BF00233139

Gibson, J. A., Cromer, L., Agius, J. T., McInnes, S. J., and Marley, N. J. (2007). Tardigrade eggs and exuviae in Antarctic lake sediments: insights into Holocene dynamics and origins of the fauna. *Journal of Limnology* **66**, 65–71. doi:10.4081/jlimnol.2007.s1.65

Guidetti, R., Schill, R. O., Bertolani, R., Dandekar, T., and Wolf, M. (2009). New molecular data for tardigrade phylogeny, with the erection of *Paramacrobiotus* gen. nov. *Journal of Zoological Systematics and Evolutionary Research* **47**, 315–321. doi:10.1111/j.1439-0469.2009.00526.x

Hebert, P. D. N., Cywinska, A., Ball, S. L., and deWaard, J. R. (2003*a*). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **270**, 313–321. doi:10.1098/rspb.2002.2218

Hebert, P. D. N., Ratnasingham, S., and deWaard, J. R. (2003*b*). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **270**, S96–S99. doi:10.1098/rsbl.2003.0025

Hengherr, S., Reuner, A., Brummer, F., and Schill, R. O. (2010). Ice crystallization and freeze tolerance in embryonic stages of the tardigrade *Milnesium tardigradum*. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **156**, 151–155. doi:10.1016/j.cbpa.2010.01.015

Huelsenbeck, J. P., and Ronquist, F. (2001). MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755. doi:10.1093/bioinformatics/17.8.754

Huerta-Cepas, J., Dopazo, J., and Gabaldón, T. (2010). ETE: a python Environment for Tree Exploration. *BMC Bioinformatics* **11**, 24. doi:10.1186/1471-2105-11-24

Jørgensen, A., Møbjerg, N., and Kristensen, R. M. (2007). A molecular study of the tardigrade *Echiniscus testudo* (Echiniscidae) reveals low DNA sequence diversity over a large geographical area. *Journal of Limnology* **66**, 77–83. doi:10.4081/jlimnol.2007.s1.77

Kristensen, R. M. (1987). Generic revision of the Echiniscidae (Heterotardigrada), with a discussion of the origin of the family. In 'Biology of Tardigrades. Selected Symposia and Monographs UZI'. pp. 261–335.

Marley, N. J., McInnes, S. J., and Sands, C. J. (2011). Phylum Tardigrada: a re-evaluation of the Parachela. *Zootaxa* **2819**, 51–64.

Marshall, D. J., and Pugh, P. J. A. (1996). Origin of the inland Acari of continental Antarctica, with particular reference to Dronning Maud Land. *Zoological Journal of the Linnean Society* **118**, 101–118. doi:10.1111/j.1096-3642.1996.tb00221.x

McGaughran, A., Stevens, M. I., Hogg, I. D., and Carapelli, A. (2011). Extreme glacial legacies: a synthesis of the Antarctic springtail phylogeographic record. *Insects* **2**, 62–82. doi:10.3390/insects2020062

McInnes, S. J. (1995). Taxonomy and ecology of tardigrades from Antarctic lakes. Master's thesis, Open University.

McInnes, S. J. (2010). *Echiniscus corrugicaudatus* (Heterotardigrada; Echiniscidae) a new species from Ellsworth Land, Antarctica. *Polar Biology* **33**, 59–70. doi:10.1007/s00300-009-0684-4

McInnes, S. J., and Pugh, P. J. A. (1998). Biogeography of limno-terrestrial Tardigrada, with particular reference to the Antarctic fauna. *Journal of Biogeography* **25**, 31–36. doi:10.1046/j.1365-2699.1998.251176.x

Michalczyk, Ł., Wełnicz, W., Frohme, M., and Kaczmarek, Ł. (2012). Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. *Zootaxa* **3154**, 1–20.

Miller, W. R., and Heatwole, H. (1995). Tardigrades of the Australian Antarctic territories – the Mawson coast, East Antarctica. *Invertebrate Biology* **114**, 27–38. doi:10.2307/3226950

Miller, W. R., Heatwole, H., Pidgeon, R. W. J., and Gardiner, G. R. (1994). Tardigrades of the Australian Antarctic territories – the Larsemann Hills, East Antarctica. *Transactions of the American Microscopical Society* **113**, 142–160. doi:10.2307/3226642

Modica, M. V., Puillandre, N., Castelin, M., Zhang, Y., and Holford, M. (2014). A good compromise: rapid and robust species proxies for inventorying biodiversity hotspots using the Terebridae (Gastropoda: Conoidea). *PLoS One* **9**, e102160. doi:10.1371/journal.pone.0102160

Murray, J. (1910). Tardigrada, in J. Murray (ed). *British Antarctic Expedition 1907–09. Reports on the Scientific Investigations. Biology*, Vol. 1, William Heinemann, pp. 83–187.

Nkem, J. N., Wall, D. H., Virginia, R. A., Barrett, J. E., Broos, E. J., Porazinska, D. L., and Adams, B. J. (2006). Wind dispersal of soil invertebrates in the McMurdo Dry Valleys, Antarctica. *Polar Biology* **29**, 346–352. doi:10.1007/s00300-005-0061-x

Pilato, G., and Binda, M. G. (1999). Three new species of *Diphascon* of the pingue group (Eutardigrada, Hypsibiidae) from Antarctica. *Polar Biology* **21**, 335–342. doi:10.1007/s003000050370

Pilato, G., and Binda, M. G. (2001). Biogeography and limno-terrestrial tardigrades: are they truly incompatible binomials? *Zoologischer Anzeiger* **240**, 511–516. doi:10.1078/0044-5231-00061

Pilato, G., and Binda, M. G. (2010). Definition of families, subfamilies, genera and subgenera of the Eutardigrada, and keys to their identification. *Zootaxa* **2404**, 1–54.

Pons, J., Barraclough, T. G., Gomez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., Kamoun, S., Sumlin, W. D., and Vogler, A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* **55**, 595–609. doi:10.1080/10635150600852011

Posada, D., and Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818. doi:10.1093/bioinformatics/14.9.817

Powers, L. E., Freckman, D. W., and Virginia, R. A. (1995). Spatial distribution of nematodes in polar desert soils of Antarctica. *Polar Biology* **15**, 325–333. doi:10.1007/BF00238482

Pugh, P. J. A. (1993). A synonymic catalogue of the Acari from Antarctica, the sub-Antarctic islands and the Southern Ocean. *Journal of Natural History* **27**, 323–421. doi:10.1080/00222939300770171

Rambaut, A., and Drummond, A. (2009*a*). Tracer. MCMC trace analysis tool. Version 1.5. 2003–2009. Computer program distributed by the authors. Available at: http://beast.bio.ed.ac.uk [Accessed September 2013].

Rambaut, A., and Drummond, A. (2009*b*). FigTree v1. 3.1. Computer program distributed by the authors. Available at: http://tree.bio.ed.ac.uk/software [Accessed September 2013].

Robeson, M., Costello, E., Freeman, K., Whiting, J., Adams, B., Martin, A., and Schmidt, S. (2009). Environmental DNA sequencing primers for eutardigrades and bdelloid rotifers. *BMC Ecology* **9**, 25. doi:10.1186/1472-6785-9-25

Sands, C. J., Convey, P., Linse, K., and McInnes, S. J. (2008*a*). Assessing meiofaunal variation among individuals utilising morphological and molecular approaches: an example using the Tardigrada. *BMC Ecology* **8**, 7. doi:10.1186/1472-6785-8-7

Sands, C. J., McInnes, S. J., Marley, N. J., Goodall-Copestake, W. P., Convey, P., and Linse, K. (2008*b*). Phylum Tardigrada: an 'individual' approach. *Cladistics* **24**, 861–871. doi:10.1111/j.1096-0031.2008.00219.x

Smykla, J., Iakovenko, N., Devetter, M., and Kaczmarek, Ł. (2012). Diversity and distribution of tardigrades in soils of Edmonson Point (Northern Victoria Land, continental Antarctica). *Czech Polar Reports* **2**, 61–70. doi:10.5817/CPR2012-2-6

Sohlenius, B., and Boström, S. (2005). The geographic distribution of metazoan microfauna on East Antarctic nunataks. *Polar Biology* **28**, 439–448. doi:10.1007/s00300-004-0708-z

Sohlenius, B., and Boström, S. (2008). Species diversity and random distribution of microfauna in extremely isolated habitable patches on Antarctic nunataks. *Polar Biology* **31**, 817–825. doi:10.1007/s00300-008-0420-5

Sohlenius, B., Boström, S., and Hirschfelder, A. (1996). Distribution patterns of microfauna (nematodes, rotifers and tardigrades) on nunataks in Dronning Maud Land, East Antarctica. *Polar Biology* **16**, 191–200. doi:10.1007/BF02329207

Soldati, L., Kergoat, G. J., Clamens, A. L., Jourdan, H., Jabbour-Zahab, R., and Condamine, F. L. (2014). Integrative taxonomy of New Caledonian beetles: species delimitation and definition of the *Uloma isoceroides* species group (Coleoptera, Tenebrionidae, Ulomini), with the description of four new species. *ZooKeys* **415**, 133–167. doi:10.3897/zookeys.415.6623

Srivathsan, A., and Meier, R. (2012). On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. *Cladistics* **28**, 190–194. doi:10.1111/j.1096-0031.2011.00370.x

Stevens, M. I., and Hogg, I. D. (2003). Long-term isolation and recent range expansion from glacial refugia revealed for the endemic springtail *Gomphiocephalus hodgsoni* from Victoria Land, Antarctica. *Molecular Ecology* **12**, 2357–2369. doi:10.1046/j.1365-294X.2003.01907.x

Stevens, M. I., and Hogg, I. D. (2006). Contrasting levels of mitochondrial DNA variability between mites (Penthalodidae) and springtails (Hypogastruridae) from the Trans-Antarctic Mountains suggest long-term effects of glaciation and life history on substitution rates, and speciation processes. *Soil Biology & Biochemistry* **38**, 3171–3180. doi:10.1016/j.soilbio.2006.01.009

Stevens, M. I., Greenslade, P., Hogg, I. D., and Sunnucks, P. (2006). Southern hemisphere springtails: could any have survived glaciation of Antarctica? *Molecular Biology and Evolution* **23**, 874–882. doi:10.1093/molbev/msj073

Stevens, M. I., Porco, D., D'Haese, C. A., and Deharveng, L. (2011). Comment on 'taxonomy and the DNA barcoding enterprise' by Ebach (2011). *Zootaxa* **2838**, 85–88.

Swofford, D. (2002). 'PAUP* version 4.0. Phylogenetic Analysis Using Parsimony (and Other Methods).' (Sinauer Associates: Sunderland, MA.)

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739. doi:10.1093/molbev/msr121

Tumanov, D. V. (2006). Five new species of the genus *Milnesium* (Tardigrada, Eutardigrada, Milnesiidae). *Zootaxa* **1122**, 1–23.

Velasco-Castrillón, A., and Stevens, M. I. (2014). Morphological and molecular diversity at a regional scale: a step closer to understanding Antarctic nematode biogeography. *Soil Biology & Biochemistry* **70**, 272–284. doi:10.1016/j.soilbio.2013.12.016

Velasco-Castrillón, A., Page, T. J., Gibson, J. A. E., and Stevens, M. I. (2014*a*). Surprisingly high levels of biodiversity and endemism amongst Antarctic rotifers uncovered with mitochondrial DNA. *Biodiversity* **15**, 130–142. doi:10.1080/14888386.2014.930717

Velasco-Castrillón, A., Schultz, M. B., Colombo, F., Gibson, J. A. E., Davies, K. A., Austin, A. D., and Stevens, M. I. (2014*b*). Distribution and diversity of soil microfauna from East Antarctica: assessing the link between biotic and abiotic factors. *PLoS One* **9**, e87529. doi:10.1371/journal.pone.0087529

Vicente, F., Fontoura, P., Cesari, M., Rebecchi, L., Guidetti, R., Serrano, A., and Bertolani, R. (2013). Integrative taxonomy allows the identification of synonymous species and the erection of a new genus of Echiniscidae (Tardigrada, Heterotardigrada). *Zootaxa* **3613**, 557–572. doi:10.11646/zootaxa.3613.6.3

Wełnicz, W., Grohme, M. A., Kaczmarek, Ł., Schill, R. O., and Frohme, M. (2011). ITS-2 and 18S rRNA data from *Macrobiotus polonicus* and *Milnesium tardigradum* (Eutardigrada, Tardigrada). *Journal of Zoological Systematics and Evolutionary Research* **49**, 34–39. doi:10.1111/j.1439-0469.2010.00595.x

Zhang, J., Kapli, P., Pavlidis, P., and Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* **29**, 2869–2876. doi:10.1093/bioinformatics/btt499